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Oral–Aboral Axis Specification in the Sea Urchin Embryo

I. Axis Entrainment by Respiratory Asymmetry

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In embryos of indirectly developing echinoids, the secondary (oral–aboral) larval axis is established after fertilization by an as yet undiscovered process. One of the earliest manifestations of this axis is an asymmetry in mitochondrial respiration, with the prospective oral side of the embryo exhibiting a higher rate of respiration than the prospective aboral side. We show here that respiratory asymmetry can be experimentally induced within embryos by immobilizing them in tight clusters of four ("rosettes"). Within such clusters a redox gradient is established from the inside to the outside of the rosette. Vital staining of clustered embryos demonstrates that the side of the embryo facing the outside of the rosette (i.e., the most oxidizing) tends to become the oral side, while the side facing the inside tends to become the aboral side. Effective entrainment of the oral–aboral axis requires that the embryos remain immobilized in rosettes until the hatching blastula stage. To begin to investigate the molecular mechanisms underlying this effect we made use of P3A2, a transcriptional regulatory protein whose activity is spatially modulated along the oral–aboral axis. When synthetic mRNA encoding P3A2 fused to the VP16 activation domain is injected into eggs, it activates embryonic expression of a green fluorescent protein reporter gene containing a basal promoter and a single strong P3A2 target site. In embryo rosettes, such activation occurs predominantly on the outside of the rosette, suggesting that the activity of the P3A2 protein is spatially regulated by the respiratory asymmetry established by clustering the embryos. These findings are discussed with reference to earlier work on both oral–aboral axis specification and P3A2 and used to develop a testable model of the mechanism of oral–aboral axis specification in the sea urchin embryo. **© 2001 Academic Press**

Key Words: **oral–aboral axis specification; sea urchin embryos; respiration; P3A2.**

INTRODUCTION

The pluteus larva of the sea urchin is organized along two orthogonal axes of symmetry. The primary or animal–vegetal (AV) axis is initially inherent in the cytoarchitecture of the unfertilized egg and is ultimately manifested during embryogenesis by differentiation of ectoderm, endoderm, and mesoderm. The secondary or oral–aboral (OA) axis is not established until after fertilization, during cleavage of the embryo. Spatially differential gene expression patterns are initiated in the presumptive oral and aboral ectoderm territories by late cleavage (reviewed in Davidson, 1989; Coffman and Davidson, 1992; Davidson *et al.*, 1998). The oral ectoderm ultimately gives rise to the larval mouth, facial epithelium, and nervous system, while the aboral ectoderm develops into a simple squamous epithelium. More subtle spatial differentiation along the OA axis also occurs in the mesendoderm (e.g., Miller *et al.*, 1996; Ruffins and Ettensohn, 1996).

The origin of the OA axis of the sea urchin larva has been a persistent mystery. Initial specification of the OA axis occurs at apparently different times in different echinoid species (reviewed by Davidson *et al.*, 1998; Henry, 1998; Cameron and Coffman, 1999). In *Strongylocentrotus purpuratus,* the OA axis bears a predictable relationship to the first cleavage plane and must therefore be initially specified during the first cell cycle (Cameron *et al.*, 1989). However, the fact that isolated blastomeres of the four-cell embryo will each develop into a relatively normal pluteus larva

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demonstrates that the early embryo is radially equipotent around the primary axis and that any secondary axial properties established in the zygote must therefore be labile (reviewed in Horstadius, 1973). The labile nature of the presumptive OA axis is further revealed by the fact that it can be experimentally entrained in the egg or cleavage-stage embryo by various physical and chemical treatments (reviewed in Horstadius, 1973). Commitment of cells to a particular fate along the OA axis does not occur until relatively late, around the time of mesenchyme blastula or early gastrula stage (Hardin *et al.*, 1992).

While gene products with asymmetric distribution patterns orthogonal to the primary axis in the egg and early embryo have been identified (reviewed in Henry, 1998; Cameron and Coffman, 1999), their relationship to specification of the OA axis has not been established. In any case, because of the radial equipotential of the early embryo it can be inferred that territory specification along the OA axis is not dependent on the localization of any particular maternal gene product. Instead, the initial specification probably depends on physiological factors that locally modulate the activities of globally distributed maternal regulatory proteins. An example of such a protein is the transcription factor P3A2, which functions to repress expression of *CyIIIa* in the prospective oral ectoderm in early development (Hough-Evans *et al.*, 1990; Calzone *et al.*, 1991; Kirchhamer and Davidson, 1996; Bogarad *et al.*, 1998). P3A2 protein is present in the egg (Zeller *et al.*, 1995; Calzone *et al.*, 1997) and available evidence suggests that it is globally distributed in the early embryo. In addition, P3A2 protein is known to be subject to extensive posttranslational modification during early development (Harrington *et al.*, 1997). The fact that P3A2 protein is globally distributed but locally activated as a repressor of *CyIIIa* in the oral ectoderm makes it an ideal transcription factor for analyzing the physiology and molecular mechanisms that underlie specification of the OA axis (Davidson *et al.*, 1998).

One of the earliest observable physiological manifestations of the OA axis is a gradient of cytochrome oxidase activity, with the prospective oral side of the embryo exhibiting a higher rate of respiration than the prospective aboral side, possibly as early as the eight-cell stage of development (Child, 1941; Czihak, 1962, 1963). That this respiratory asymmetry might actually play a role in OA axis specification was suggested by a series of experiments performed by Daniel Pease in the 1940s. Pease (1941, 1942) showed that the OA axis could be statistically entrained by exposing cleavage-stage embryos to steep concentration gradients of dilute respiratory inhibitors such as cyanide, with the most inhibited side of the embryo tending to develop as aboral. Additional evidence that respiratory asymmetry plays a role in OA axis specification is provided by the observation that treatments which abolish respiratory asymmetry also radialize the embryo (Child, 1948; Czihak, 1962). While the respiratory asymmetry observed in the early embryo may be a downstream consequence of whatever symmetry-breaking process initially specifies the OA axis, the experimental evidence would indicate that it nonetheless underlies and is necessary for normal specification of cell fate along the OA axis. Potential mechanisms through which respiration could influence cell fate include effects of mitochondrial activity and cellular redox state on cell signaling and transcription factor activities (see e.g., Duchen, 1999; Sun and Oberly, 1996; for reviews).

We present here a new investigation of the phenomenon of OA axis entrainment by induced respiratory asymmetry. This investigation takes advantage of the fact that embryos immobilized in tight clusters of four (hereafter referred to as "rosettes") establish by means of mitochondrial respiration a redox gradient from the inside to the outside of the cluster. Within such embryo rosettes, the sides of the embryos facing out tend to become oral, while the sides facing in tend to become aboral. This entrainment of the OA axis requires that the embryos remain immobilized in the rosettes until the hatching blastula stage. To begin to address the molecular basis of this phenomenon, we develop a novel green fluorescent protein (GFP) reporter gene assay for P3A2 protein activity and with it show that P3A2 is more active on the outside of embryo rosettes than on the inside.

MATERIALS AND METHODS

Manipulation of Eggs and Embryos

Eggs and sperm were obtained from *S. purpuratus* by either vigorous shaking or mild electric shock. Eggs were dejellied by a short incubation in acidic (pH ~4.8) filtered seawater, followed by two washes in filtered seawater (FSW). Protamine sulfate (PS)-coated petri dishes were prepared as described previously (McMahon *et al.*, 1985). A finely drawn mouth pipette was used to arrange the eggs in tight clusters of four (rosettes) on the bottoms of the PS-coated dishes (see Figs. 1 and 3 for typical rosettes), in FSW containing 0.1 mg/ml 3-amino-1,2,4-triazole (Sigma). The clustered eggs were fertilized with a dilute sperm suspension and in some cases either injected immediately with DNA and/or RNA as described previously (McMahon *et al.*, 1985; Kirchhamer and Davidson, 1996) or labeled with Nile blue as described below. In other cases the fertilized eggs were allowed to develop until the four-cell stage, at which time they were either labeled with Nile blue or stained with Redoxsensor red (Molecular Probes, Eugene, OR) as described below. Injected or labeled embryos were observed at the four-cell stage, and any that were found to have serious cleavage defects or which were not optimally oriented (i.e., those in which the AV axis was parallel to the in–out axis of the embryo with respect to the rosette) were discarded. Injected embryos were viewed and imaged at the 16-h blastula stage on a Zeiss/CARV fluorescence microscope equipped with a Quantix cooled CCD digital camera.

Nile blue labeling was performed as follows. A 1% solution of Nile blue was prepared in molten 0.5% agarose, drawn by suction into the ~25- to 50-μm diameter open tip of a microinjection needle, and allowed to solidify by cooling. A Narishige micromanipulator was used to position the Nile blue tip on the surface of a fertilized egg or four- to eight-cell stage embryo for 5–60 s, resulting in a blue spot 0.25–0.5× the diameter of the embryo. The extent of staining was monitored visually through a dissecting microscope.

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Stained embryos were allowed to develop within rosettes for varying lengths of time until hatching, after which they were collected with a mouth pipette and transferred to ~100-μm-diameter agar tunnels (Ransick and Davidson, 1995) in FSW containing penicillin (20 units/ml) and streptomycin (50 μg/ml). The embryos developed in the agar tunnels until late gastrula stage (~48 h postfertilization), at which time the position of the Nile blue mark with respect to the OA axis was recorded.

Embryos were stained with Redoxsensor red and in some cases with Mitotracker green (both reagents from Molecular Probes) by replacing the FSW in the culture dish with FSW containing 1 μM Redoxsensor Red CC-1 or 200 nM Mitotracker green, followed by a 10-min incubation in the dark at 15°C. The embryos were then washed with four or five changes of FSW, viewed, and imaged as described above for injected embryos. Embryo images were processed in Adobe PhotoShop 5.0.

Preparation of DNA Constructs and Synthetic mRNA

The *SM3a2EpGFP* reporter gene was prepared by annealing the oligonucleotide pair SM3a2+, GATCTTTTCGGCTTCTGCGCACACCCCACGCGCATGGGGC, and SM3a2−, GATCGCCCCATGCGCGTGGGGTGTGCGCAGAAGCCGAAAA (Xian *et al.*, 1996), and inserting them into the *Bgl*II site in the polylinker upstream of the *Endo16* basal promoter in the basic gene-trap vector *EpGFPII* (A. Cameron, P. Oliveri, J. Wyllie, and E. Davidson, manuscript in preparation; see Fig. 3A). Plasmids containing the SM3a2 oligonucleotide insert in both orientations were obtained and found in preliminary experiments to be equally responsive to P3A2-VP16. However, only those with the SM3a2 insert in the + orientation with respect to the GFP coding sequence were used in these studies. The construct *HEGFP*, which contains the green fluorescent protein coding sequence under the control of the spatial *cis*-regulatory domain of *SpHE* (Wei *et al.*, 1995, 1997), was described previously (Bogarad *et al.*, 1998). Both constructs were linearized with *Kpn*I for microinjection.

The P3A2-VP16 fusion construct was prepared as follows. The coding sequence of P3A2 was amplified by polymerase chain reaction (PCR) using Elongase (Gibco BRL) and the following primers: P3A2wt+, GAAGATCTCAATATGATGATCAGTGAAGATATCAGTGAGCCGTCCTCCCCGGAC, and P3A2−, GCTCTAGACGTCTGTATGTTGATCATGTGATGCTGCATTGAC. The resulting amplicon was digested with *Xba*I and *Bgl*II, purified by agarose gel electrophoresis, and cloned into the same sites in the vector pHET7. The resulting construct pHET7-P3A2 contains a T7 promoter, followed by the entire P3A2 ribosome binding and coding sequences with an *Aat*II site inserted immediately upstream of the stop codon, and an SV40 polyadenylation signal. The VP16 activation domain was amplified from the pTet-Off vector (Clontech) by PCR using Elongase (Gibco BRL) and the following primers: VP16ad+, CCGACGTCTCCGCGTACAGCCGCGCGCGTA, and VP16ad−, CCGACGTCCTACCCACCGTACTCGTCAATTCCAAG. The resulting amplicon was digested with *Aat*II, purified by gel electrophoresis, and cloned into the *Aat*II site of pHET7P3A2, resulting in pHET7-P3A2VP16 (see Fig. 3A). The latter plasmid was linearized with *Bam*HI and used as a template to synthesize capped mRNA using the T7 mMessage mMachine (Ambion).

RESULTS

Embryos Clustered in Rosettes Establish a Redox Gradient from the Inside to the Outside of the Rosette

Our preliminary experiments using the indophenol blue reaction to visualize cytochrome oxidase activity in the early embryo (Child, 1941; Czihak, 1963) indicated that when embryos are clustered together, a dye reduction and/or cytochrome oxidase activity gradient is established from the inside to the outside of the cluster (data not shown). In order to investigate this phenomenon further, eggs were immobilized in rosette clusters on protamine sulfate-coated dishes, fertilized, and, at the four-cell stage, stained with 2,3,4,5,6-pentafluorodihydrotetramethylrosamine (Redoxsensor red), a fluorescent indicator of cellular redox state (Chen *et al.*, 1998). To control for dye penetration artifacts, the same embryos were also stained with Mitotracker green, a fluorescent probe that stains mitochondria irrespective of cellular redox state. Figure 1A depicts an embryo rosette stained with both fluorescent probes: the green stain indicates mitochondrial distribution, while the red stain is indicative of cellular oxidation. The overlap between the two stains appears yellow-orange. As can be seen in this figure, a redox gradient is established in the embryo rosettes, such that the blastomeres facing out are more oxidizing than the blastomeres facing in.

The most obvious explanation for the observed redox gradient is that an oxygen gradient is established across the embryos in the rosette as a result of respiratory competition in the center. To test this hypothesis, embryos were treated with 1 mM KCN to block mitochondrial respiration and then stained with Redoxsensor red and Mitotracker green. As shown in Fig. 1C, such treatment abolishes the redox gradient within the rosette (compare with controls in Fig. 1B), as would be expected if the gradient were the result of mitochondrial respiration. These results demonstrate that immobilizing embryos in rosettes is an effective way to generate respiratory asymmetry with a predictable orientation, that is, across each embryo from the inside to the outside of the rosette.

The Oral–Aboral Axis Is Entrained in Embryo Rosettes

To test whether clustering embryos in rosettes has any effect on development of the oral–aboral axis, eggs were immobilized in rosettes on protamine sulfate-coated dishes, fertilized, and, at the four-cell stage, marked on either the outer or the inner blastomere with Nile blue (Fig. 2A). In this experiment, only embryos that were oriented with the AV axis more or less orthogonal to the inside–outside (IO) axis of the rosette were labeled. At late gastrula stage the position of the label was scored with respect to the OA axis. Since the label tended to fall over 1/3–1/2 of the embryo, it was scored as either oral or aboral (e.g., oral-lateral was scored as oral and vice versa), unless the label was equally distributed between the two territories, in which case it

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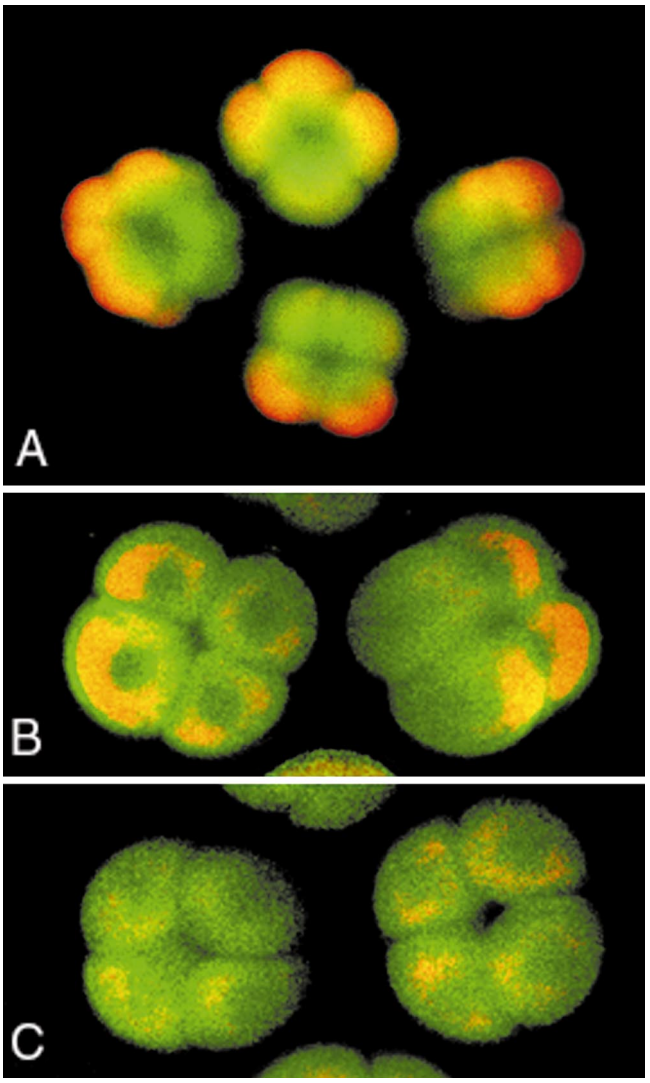

FIG. 1. Respiratory redox asymmetry is established in embryos immobilized in clusters of four (rosettes). (A) Clustered eight-cell embryos stained with both Redoxsensor red (shown in red), which is indicative of cellular oxidation, and Mitotracker green (shown in green), which is indicative of mitochondrial distribution. The yellow-orange color corresponds to the overlap between the two stains. (B) Two four-cell embryos within a rosette stained with Redoxsensor red and Mitotracker green. (C) Two four-cell embryos within a rosette treated with 1 mM KCN and then stained with Redoxsensor red and Mitotracker green.

was scored as lateral. As can be seen in Fig. 2B and in Table 1 (lines 1 and 2), when the labeled embryos were allowed to develop within rosettes until hatching, a correlation was observed between the orientation of the OA axis of the embryo and the IO axis of the rosette. In a statistically significant fraction of cases, the side of the embryo facing the outside of the rosette developed as the oral side, while the inside developed as the aboral side (Table 1). Since the rate of respiration in the middle of each rosette is lower than on the outside, these results are in agreement with the experiments of Pease (1941, 1942), wherein the side of the embryo exposed to the highest concentration of respiratory inhibitor tended to develop as aboral.

Oral–Aboral Axis Entrainment Requires That Embryos Remain in Rosettes until Hatching

The entrainment of the OA axis apparent in Fig. 2 and Table 1 was achieved by allowing embryos to develop within rosettes until they hatched, at which point they swam away freely. We next sought to determine the minimum length of time that embryos must remain in a rosette for OA axis entrainment to occur. To this end, embryos were clustered and labeled as in the experiments described above and at various times prior to hatching were removed from the clusters and allowed to develop to the late gastrula stage, when the position of the Nile blue mark was re-

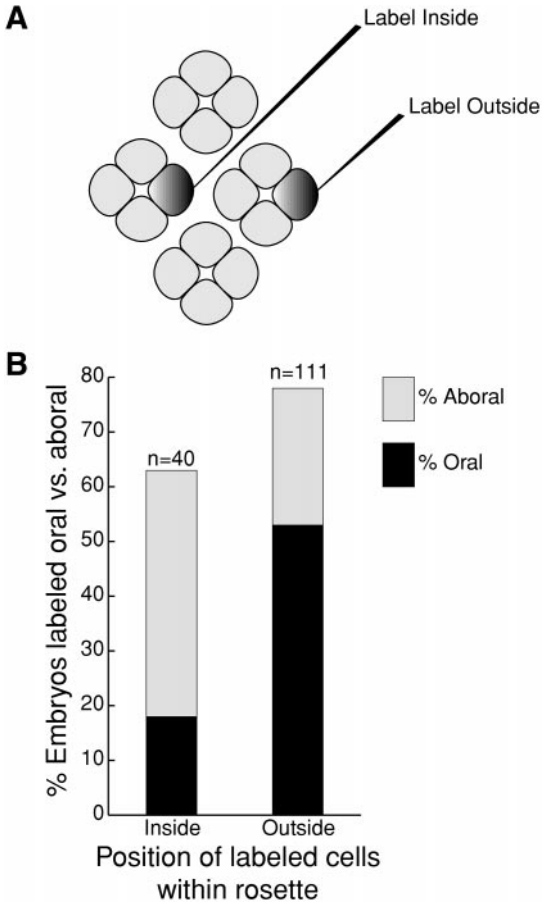

FIG. 2. The oral–aboral axis is entrained in embryos clustered in rosettes. (A) Diagram depicting the Nile blue labeling procedure used in these experiments. (B) Graphical representation of the data listed in Table 1.

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TABLE 1

Oral–Aboral Axis Development in Embryos Clustered in Rosettes[a]

Label position[b]	Hpf removed from rosette	Total number scored	Number labeled oral[c]	Number labeled aboral[c]	Number labeled lateral[c]	Oral/aboral labeling ratio	Significance[d]
Inside	16 (HB)	40	7	18	15	.39	$P < 0.05$
Outside	16 (HB)	111	59	28	24	2.11	$P \ll 0.01$
Outside	12 (EB)	74	36	27	11	1.33	NS
Outside	6–8 (C)	87	36	31	20	1.16	NS

[a] Results were compiled from 10 different experiments. Embryos immobilized in rosettes were labeled with Nile blue at fertilization (two experiments) or the four-cell stage (eight experiments), at the indicated positions with respect to the rosette. The embryos remained immobilized in the clusters for the indicated times and were scored for the position of the Nile blue label at late gastrula stage (approximately 48 h postfertilization). Abbreviations: Hpf, hours postfertilization; HB, hatching blastula; EB, early blastula; C, cleavage stage; NS, not significant.

[b] For explanation see Fig. 2A.

[c] Scoring of labeled embryos was performed with respect to the plane that bisects the OA axis, as follows. When the majority of label was within the oral half of the embryo it was scored as oral. When the majority of label was within the aboral half of the embryo it was scored as aboral. When the label was distributed equally between oral and aboral halves, or was largely animal (acronal) or vegetal (endodermal) or otherwise not obviously oral or aboral, it was scored as lateral. Abnormally developing embryos were not scored.

[d] Significance was calculated using a χ^2 test with 1 degree of freedom, by counting only those embryos whose label could be assigned to the categories oral or aboral. The null hypothesis is that for any embryo in which the label is not scored as lateral, there is an equal (50%) probability that label will be scored as either oral or aboral.

corded. The results of these experiments are summarized in Table 1 (lines 3 and 4). When embryos were removed from the rosettes prior to hatching, either during cleavage (6, 7, or 8 h postfertilization) or as late as early blastula stage (12 h postfertilization), there was only a slight and statistically insignificant bias in the orientation of the OA axis. This suggests either that OA axis entrainment is a cumulative process that requires prolonged incubation within a rosette or that the effective period for entrainment is relatively late (i.e., blastula stage). These results are consistent with previous studies (e.g., Hardin *et al.*, 1992) indicating that while OA axial properties may be initially specified during early cleavage, the axis itself is not *determined* until much later, around the time of late blastula stage. Finally, these data rule out a plausible alternative explanation for the entrainment effect, i.e., that clustering embryos causes OA axis entrainment by limiting the egg surface area available for sperm entry, which could conceivably bias the location of sperm aster formation within the zygote. Such an explanation would require that significant entrainment occur even in embryos removed from rosettes shortly after fertilization.

P3A2-VP16 Protein Activity Is Spatially Modulated in Embryo Rosettes

To begin to address the molecular mechanism through which clustering of embryos affects development of the OA axis, we developed a reporter system for visualizing the DNA-binding activity of the transcription factor P3A2 in living embryos. P3A2 is a repressor of *CyIIIa* in the oral ectoderm (Bogarad *et al.*, 1998, and references therein) that is present as a globally distributed maternal protein in the early embryo. The P3A2 protein is subject to posttranslational modifications that locally activate it in the oral ectoderm (Kirchhamer and Davidson, 1996; Harrington *et al.*, 1997; Davidson *et al.*, 1998). In order to visualize P3A2 DNA-binding activity directly in living embryos, the coding sequence of P3A2 was fused in frame to that of the VP16 activation domain, and the resulting construct was used to make synthetic capped mRNA for injecting into sea urchin eggs (Fig. 3A). The reporter gene (*SM3a2EpGFP*) encodes green fluorescent protein under the control of a single strong P3A2 target site (derived from the *SM50* gene; Calzone *et al.*, 1991; Xian *et al.*, 1996; see Fig. 3A) driving a basal promoter that has no inherent spatial expression bias in the embryo (the basal promoter of the *Endo16* gene; Yuh *et al.*, 1996, 1998; Yuh and Davidson, 1996). Preliminary experiments demonstrated that the *SM3a2EpGFP* reporter is not activated by endogenous P3A2, but is activated in blastula-stage embryos by excess P3A2-VP16 made from synthetic mRNA injected into eggs. Furthermore, this activation requires the presence of the P3A2 target site in *SM3a2EpGFP*, since the parent plasmid *EpGFP* (which lacks this site) is not activated by P3A2-VP16. Thus, in a control experiment in which P3A2-VP16 mRNA was injected at ~0.1 pg mRNA/egg, or $\sim 10^5$ molecules/egg (the highest mRNA concentrations used in these experiments), 82/102 embryos expressed co-injected *SM3a2EpGFP*, while 0/91 embryos expressed co-injected *EpGFP*. The minimum amount of injected P3A2-VP16 mRNA required to achieve activation of the *SM3a2EpGFP* reporter in the 15-h blastula was $\sim 10^4$ copies per egg, or approximately 10-fold excess over endogenous P3A2 mRNA (Cutting *et al.*, 1990; Zeller *et al.*, 1995). Assuming a translation rate of two protein molecules $mRNA^{-1}$ min^{-1} and a lag time of 1 h to allow for

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TABLE 2
Spatial Activation of *SM3a2EpGFP* by P3A2-VP16 in Embryo Rosettes

P3A2-VP16 [mRNA] injected (ng/μl)	Total number expressing GFP	Number expressing outside (O)[a]	Number expressing inside (I)[a]	Number expressing both O+I[a]	O/I expression ratio	Significance[b]
50	60	23	14	23	1.6	NS
25	37	14	5	18	2.8	$P < 0.05$
12.5	50	25	10	15	2.5	$P \approx 0.01$
≤25 (totals from above)	*87*	*39*	*15*	*33*	*2.6*	$P \ll 0.01$
− (*HEGFP* control)	*85*	*15*	*12*	*58*	*1.25*	NS

[a] Results are combined from six different experiments, examples of which are depicted in Fig. 3. Scoring of embryos expressing GFP was performed with respect to the plane that bisects the axis running through each embryo from the outside to the inside of the cluster (see Fig. 3B), as follows. When all of the GFP was detected within the outside half of the embryo, it was scored as outside (O). When all of the GFP was detected within the inside half of the embryo, it was scored as inside (I). Otherwise, the embryo was scored as expressing in both domains (O+I). The data in rows 4 and 5 (italics) compare the combined results from rows 2 and 3 with the results from the control injections of *HEGFP*.

[b] Significance was calculated using a χ^2 test with 1 degree of freedom, by counting only those embryos that could be assigned to the categories O or I. The null hypothesis is that for any embryo not scored as O+I there is an equal (50%) probability that GFP will be expressed entirely within the O or I domains. NS, not significant.

ribosome loading, such a quantity of injected mRNA would be expected to generate $\sim 2 \times 10^7$ protein molecules by 15 h of development (Zeller *et al.*, 1995; Davidson, 1986). This is only ~10 times the amount of endogenous P3A2 at this stage, which is not unreasonable, given that P3A2-VP16 must compete with endogenous P3A2 for target site occupancy to achieve reporter gene activation. Moreover, the actual amount of P3A2-VP16 protein per embryo in these experiments is probably somewhat less than that given by this estimate, since it assumes zero protein turnover.

P3A2-VP16 mRNA and SM3a2EpGFP DNA were coinjected into zygotes immobilized in rosettes, and the spatial expression of GFP recorded at the blastula stage just prior to hatching (15–16 h postfertilization). *HEGFP*, which is normally expressed throughout the ectoderm, was used as a control for spatial expression patterns that might result from mosaicism and/or injection artifacts. Several examples of *SM3a2EpGFP* expression in embryo rosettes are depicted in Figs. 3C–3J and the combined results of several experiments are summarized in Table 2. When P3A2-VP16 mRNA was injected at concentrations just above the minimum required for activation of the reporter, GFP was expressed in approximately 70% of injected embryos and in those approximately 2.5 times more often in the outside than in the inside of the rosette (Table 2). These data indicate that the activity of the P3A2-VP16 protein is spatially modulated along the IO axis of the rosette, since P3A2-VP16 mRNA would be expected to be everywhere in these embryos. Since embryos injected with P3A2-VP16 mRNA fail to gastrulate (not shown), the expression of *SM3a2EpGFP* could not be observed at stages in which the OA axis could be morphologically distinguished. However, the 2.5-fold predominance of *SM3a2EpGFP* expression on the outside of the rosette is roughly equivalent to the predominance of oral to aboral development on the outside (shown in Fig. 2 and summarized in Table 1), suggesting that the former is related to the latter. These data are also consistent with the fact that endogenous P3A2 is more active in the oral ectoderm than in the aboral ectoderm (Kirchhamer and Davidson, 1996).

DISCUSSION

Oral–Aboral Axis Specification in the Sea Urchin Embryo

The mechanism underlying OA axis specification in the sea urchin embryo remains unknown, despite a large body of relevant experimental data (reviewed in Horstadius, 1973; Davidson *et al.*, 1998; Henry, 1998). The objective of this and of other ongoing studies is to elucidate that mechanism. Toward that end, a testable model that accounts for all of the experimental data must be developed. The observations that require explanation are as follows:

(1) The OA axis bears a predictable relationship to the plane of first cleavage in some echinoid species (e.g., *S. purpuratus*), but not in others (e.g., *Lytechinus variegatus;* reviewed in Henry, 1998; Davidson *et al.*, 1998).

(2) Spatially differential zygotic gene expression along the OA axis of the embryo is initiated early in development, during cleavage (reviewed in Coffman and Davidson, 1992; Davidson *et al.*, 1998).

(3) The OA axis is labile and can be experimentally entrained (as shown here), duplicated, or abolished (reviewed in Horstadius, 1973).

(4) The OA axis is not irreversibly determined until late blastula or early gastrula stage (Hardin *et al.*, 1992).

Taken together, these observations lead to the conclusion

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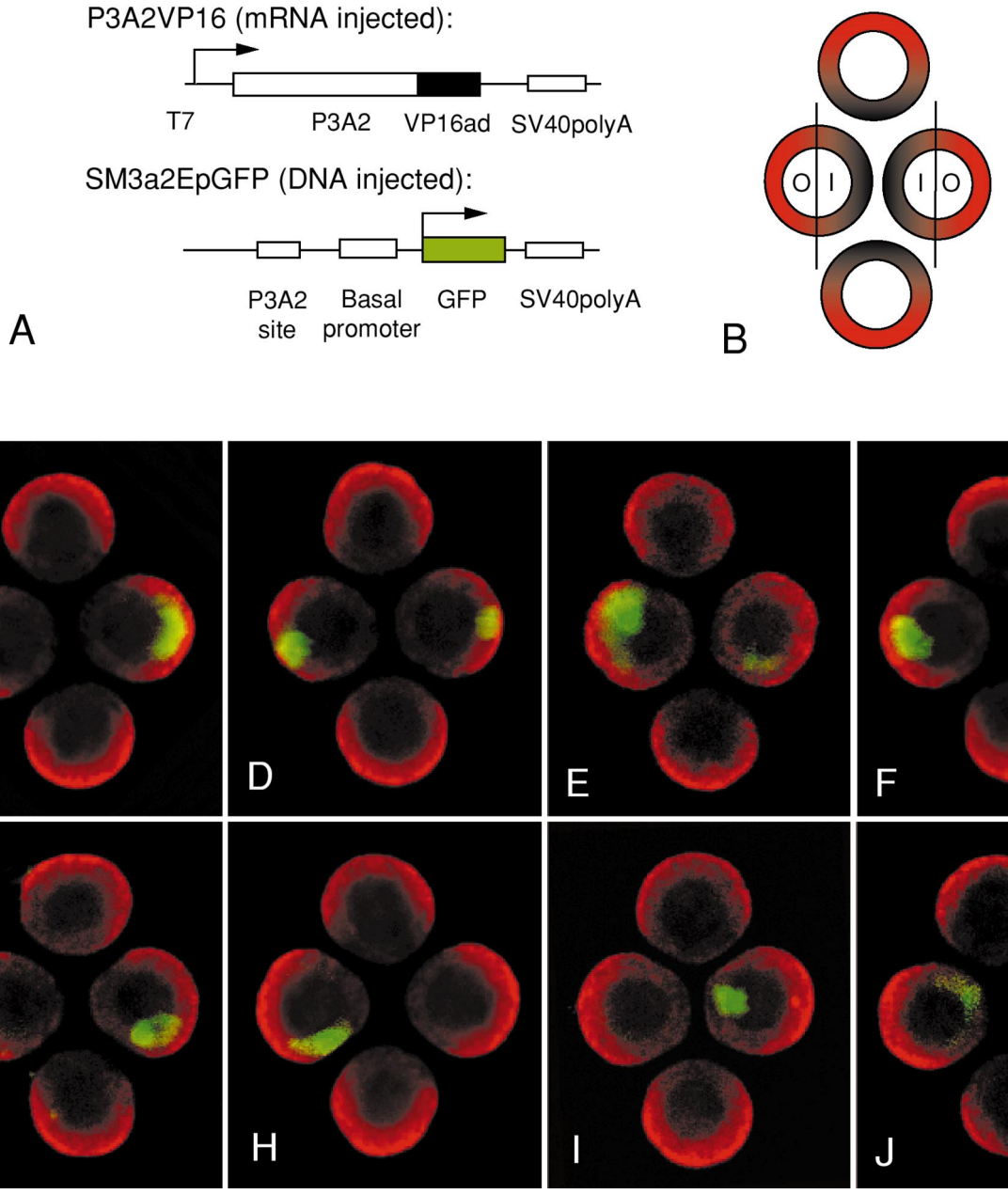

FIG. 3. The activity of a P3A2-VP16 fusion protein is spatially modulated in embryo rosettes. (A) Diagram depicting the molecular constructs used in these experiments. (B) Diagram depicting the method used for scoring GFP expression in embryo rosettes, wherein the embryos are visually partitioned into inside ("I") and outside ("O") spatial domains. (C–J) Examples of blastula stage embryo rosettes expressing *SM3a2EpGFP* under the control of P3A2-VP16 translated from mRNA injected into eggs. C–F show five embryos expressing GFP in the O domain and one embryo expressing GFP in the O+I domain (E); G and H show two embryos expressing in the O+I domain; and I and J show two embryos expressing in the I domain and one embryo expressing in the O+I domain (J). The embryos are stained with Redoxsensor red for contrast.

that gene regulatory processes underlying spatial specification of cell fate along the embryonic OA axis begin during cleavage, yet remain quite plastic and are not completed until sometime during gastrulation. A molecular explanation for this is that differential gene expression along the OA axis of the embryo initially depends on globally distributed maternal transcription factors, the activities of which are spatially modulated by posttranslational modification

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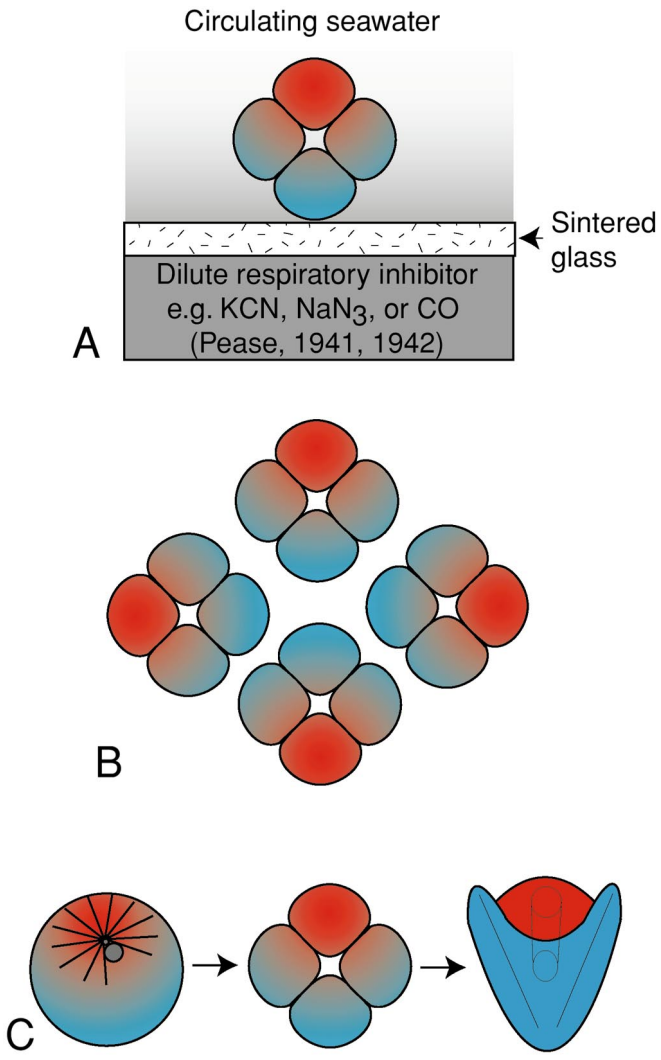

FIG. 4. Summary of different experimental methods used to entrain the oral–aboral axis by respiratory asymmetry, and a proposed model to account for the results. (A) The experimental setup of Pease (1941, 1942) in which exposure of embryos to steep concentration gradients of dilute respiratory inhibitors was found to entrain the oral–aboral axis in *Dendraster.* (B) The experimental setup used here. Embryos clustered together in rosettes establish a respiratory/redox gradient from the inside to the outside of the rosette. As in the experiments of Pease illustrated in A, the oral pole of the embryo develops on the side with the highest respiratory activity. (C) A proposed model for oral–aboral axis specification. Subtle respiratory asymmetry generated in the early zygote, possibly via cytoskeletal activity (e.g., sperm aster formation), persists through cleavage, which partitions the asymmetry into different blastomeres, wherein the activities of transcriptional regulatory proteins are differentially modulated. The pole of the early embryo with the highest respiratory activity ultimately develops into oral ectoderm.

in the early embryo (Davidson, 1989; Coffman and Davidson, 1994; Davidson *et al.,* 1998). This may occur as a result of physiological asymmetries in the egg or zygote and/or in response to intercellular signaling. The major questions raised by this explanation are what are the transcriptional regulatory proteins that initially control spatially differential gene expression along the OA axis and what is the source of the spatial information to which these proteins respond? The experiments described here suggest that at least some of the relevant spatial information is provided by mitochondrial respiration.

Respiratory Asymmetry and Entrainment of the OA Axis

By exposing cleavage-stage embryos to steep concentration gradients of dilute respiratory inhibitors, Pease (1941, 1942) was the first to demonstrate that the OA axis of echinoid embryos can be entrained by induced respiratory asymmetry (Fig. 4A), with the oral pole developing on the least inhibited side of the embryo. Here we have used a different experimental method to achieve a similar effect (Fig. 4B). As shown in Fig. 1 of this paper, clustering of embryos in rosettes establishes a redox gradient across each embryo in the rosette as a consequence of mitochondrial respiration. Nile blue labeling experiments show that in embryos that develop within such rosettes until hatching, the oral side of the embryo tends to develop on the side facing out of the rosette, i.e., the side with the highest respiratory activity. Since the experiments of Pease (1941, 1942) clearly demonstrate that respiratory asymmetry per se causes OA axis entrainment, the simplest interpretation of our results is that the respiratory asymmetry generated within the embryo rosettes causes the observed entrainment of the OA axis. However, we cannot at this point rule out the possibility that factors in addition to the induced respiratory asymmetry (e.g., concentration gradients of metabolites within the rosettes) also play a role in the entrainment effect.

As in the experiments of Pease, the entrainment effect described here is never 100% complete, suggesting that the axial information provided by imposed respiratory asymmetry must overcome endogenous axial information initially set up in the zygote, for example, asymmetries in mitochondrial distribution. This is underscored by the fact that the embryos must remain immobilized in rosettes through hatching for significant entrainment to occur. The most likely explanation for the entrainment is that the respiratory asymmetry established within the rosette cumulatively affects the spatial activities of transcriptional regulatory proteins involved in OA axis specification, e.g., via effects on intracellular redox state and/or signaling through reactive oxygen species. We have shown here that the transcription factor P3A2 is likely to be one of the proteins whose activity is modulated by respiratory activity.

P3A2 and OA Axis Specification

P3A2 was originally identified as a protein that represses *CyIIIa* expression in the oral ectoderm (Hough-Evans *et al.,*

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1990; Calzone *et al.*, 1991). It was subsequently demonstrated that P3A2 functions as a repressor within the proximal module of the *CyIIIa* regulatory domain, which is required for early spatial regulation of *CyIIIa* expression (Kirchhamer *et al.*, 1996). This, together with the fact that it is present as a maternal protein subject to extensive posttranslational modification (Zeller *et al.*, 1995; Calzone *et al.*, 1997; Harrington *et al.*, 1997), indicates that P3A2 is one of the transcriptional regulatory proteins that transduce axial information provided by the zygote into spatially differential gene expression (Davidson *et al.*, 1998). To analyze this we have developed a novel P3A2-VP16-activated GFP reporter system for observing P3A2 DNA binding activity directly in living embryos. Using this system, we have shown here that P3A2 DNA binding activity is responsive to the asymmetry established by clustering embryos in rosettes (Fig. 3 and Table 2), being more active on the outside of the rosette than on the inside.

In this regard it is interesting that P3A2 is highly similar to its mammalian homologue, NRF-1 (nuclear respiratory factor 1; Virbasius *et al.*, 1993). The latter protein was originally identified as an activator of numerous mitochondrial genes encoded in the nucleus, such as cytochrome *c*. Because of its high similarity to NRF-1, it would not be surprising if P3A2 was also involved in regulating the expression of mitochondrial genes, a possibility that we are investigating. It has also been shown that enhanced mitochondrial respiration up-regulates the activity of NRF-1 (Li *et al.*, 1999) and that the DNA binding activity of NRF-1 is enhanced by phosphorylation of a group of five N-terminal serines (Gugneja and Scarpulla, 1997). Four of these five serines are conserved in P3A2. We have obtained evidence that the phosphorylation of these serines enhances P3A2 DNA binding activity *in vitro* and that this phosphorylation is redox sensitive (unpublished observations). The intriguing possibility is thus raised that P3A2, like NRF-1, both regulates and is regulated by mitochondrial activity and that this positive feedback circuit plays a role in OA axis specification.

A Model for OA Axis Specification

A plausible explanation for the predictable relationship between the first cleavage plane and the orientation of the OA axis in species such as *S. purpuratus* is that the cytoskeletal rearrangements associated with fertilization provide spatial information used to specify the axis. As proposed by Cameron *et al.* (1989), these cytoskeletal rearrangements could result in a localized accumulation of mitochondria, which would account for the early asymmetries in cytochrome oxidase observed by Czihak (1963). In addition, the sperm aster is a particularly active region of cytoplasm in the zygote, involving a number of ATP-dependent processes. In the early sea urchin embryo, mitochondrial activity is repressed by a high ATP/ADP ratio (Fujiwara and Yasumasu, 1997; Fujiwara *et al.*, 2000), so the localized ATP sink provided by the sperm aster would be expected to stimulate a localized increase in mitochondrial respiration. This in turn would result in a localized increase in [ATP], which by diffusion would contribute to long-range inhibition of respiration in other regions of the single-cell zygote.

A model for OA axis specification that emerges from these considerations is as follows (Fig. 4C). The oral pole of the OA axis may initially be specified by cytoskeletal activities associated with fertilization, which by way of localized ATP consumption and/or accumulation of mitochondria could generate a subtle yet persistent respiratory asymmetry in the early zygote. Respiratory asymmetry could also arise stochastically by any number of alternative mechanisms in the early embryo, which would account for the dissociation between cleavage and OA axis specification observed in some species and also for the highly regulative nature of OA axis specification. With cleavage, the respiratory asymmetry becomes partitioned into different blastomeres, wherein the activities of transcription factors are differentially modulated (e.g., directly or indirectly via respiratory effects on cellular redox state, calcium dynamics, kinase activities, etc.). A key transcription factor in this process is P3A2, which is up-regulated by enhanced mitochondrial activity on the presumptive oral side of the embryo and which is predicted to play in turn a role in positively regulating mitochondrial activity. This would result in the generation of a positive feedback circuit that would amplify the initial subtle respiratory asymmetry, which together with intercellular signaling (Wilt, 1987; Davidson, 1986, 1989; Davidson *et al.* 1998; Wikramanayake and Klein, 1999) would lead to progressive determination of cell fate along the OA axis through early development. The predictions of this model are that (1) a locus of enhanced respiration specifies the prospective oral pole in the zygote, (2) P3A2 activity is stimulated by elevated mitochondrial activity on the prospective oral side of the embryo, (3) P3A2 plays a positive role in regulating mitochondrial gene batteries, and (4) P3A2 regulates the expression of other transcriptional regulatory genes involved in cell fate specification along the OA axis. In this paper, we have provided evidence that supports predictions (1) and (2). With current technology, it should be relatively straightforward to test the other predictions as well and in the near future to solve the long-standing problem of oral–aboral axis specification in the sea urchin embryo.

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